THE EFFECTS OF POSTNANTAL HYPERTHYROIDISM ON CORONAL SUTURE FUSION AND CALVARIAL GROWTH IN RABBITS WITH FAMILIAL, DELAYED-ONSET SYNOSTOSIS: TESTING A GENE/ENVIRONMENTAL CAUSAL MECHANISM OF VARIABLE PHENOTYPIC EXPRESSION OF CRANIOSYNOSTOSIS

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Early fusion of craniosynostosis may occur prior to birth or afterwards during cranial vault growth. The cause of craniosynostosis may be due to genetic maturations and/or environmental influences. Many reports show environmental influences increasing the penetrance or expression of craniosynostosis. The purpose of this study is to identify the environmental influences of post-natal administration of tri-iodothyronine (T3) in rabbits that demonstrate familial delayed onset craniosynostosis, and identify a possible gene/environment interaction.

A total of 65 New Zealand white rabbits (Oryctolagus cuniculus) were used. Forty one (41) rabbits with delayed onset craniosynostosis were obtained, as well as 24 in-colony normal rabbits from a similar colony, but who did not display phenotypic expression of craniosynostosis. Both phenotypic groups were divided into 3 treatments groups: post-natal injections of tri-iodothyronine (T3), vehicle sham, or control no-treatment group. A 2x3x3 (phenotype x treatment x age) design was used. Amalgam markers were placed around frontonasal, coronal and anterior lambdoid suture. Lateral and dorsoventral cephalograms were taken at 10, 25 and 42 days. Lateral cephalograms were traced, 13 anatomic landmarks identified, and both linear and angular measurements were made. Correlation between treatment group and coronal suture marker measurement was made.

Results showed statistically increased blood T3 levels ($F = 5.96$, $p<0.005$) and decreased blood T4 levels ($F = 41.07$; $p<0.000$) in T3 treated groups. Coronal suture marker separation

decreased in treated rabbits but was not significant (F=2.07; p NS). Mean changes in body weight were significant in T3 treated groups (F=6.91; p<0.002). Mean changes in total craniofacial length, cranial vault and length, and cranial base angle were not significant. Mean changes in cranial vault shape index were significant (F=5.837; p<0.006). Mean changes in palatal angle were significant for delayed on-set rabbits as well as T3 treated groups (F=4.535; p<0.05; and F=3.333; p<0.05).

In conclusion, the effect of T3 on cranial development showed changes in cranial vault shape index and palatal plane angle. Decreased coronal suture marker separation was observed in T3 treated rabbits but not statistically significant. No gene/environment interaction was observed in this study and other factors affecting variable phenotypic expression should be explored.

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PREFACE

To my wife, Jennifer Dawn Feller, who has supported me whole-heartedly. Thank you for the countless days you've spent raising our children, putting great bread on the table, and keeping our family together. When I think of what you've given up for me, and for our family, I feel truly humbled. If one day you want to go to school for 7 years, I'd gladly let you:) I love you with all my heart, and I am always yours!

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1.0 INTRODUCTION

1.1 CRANIOSYNOSTOSIS

Craniosynostosis is defined as early fusion of one or more of the cranial sutures (Cohen et al. 1993). Early cranial suture fusion may occur prior to birth (known as early on-set) or post-natally (known as delayed on-set synostosis) (Reddy et al. 1990; Cohen et al. 1993) inhibiting the growth of the skull and brain (Babler and Persing, 1982; Marsh and Vannier, 1985; Richtsmeier et al. 1991; Kreiborg, 1986; Sarnat, 1989). Understanding cranial sutures and their development may increase awareness of clinical problems and their approaches for treatment.

The cranial vault, or neurocranium, undergoes intramembranous ossification from the mesenchymal tissues, and consists of part of the temporal, frontal, parietal, and portions of the occipital, ethmoid, and sphenoid bones. Although individual, they are adjoined to one another by fibrous connective tissue. The fibrous connective tissue junctions are called sutures, and in the head, are called cranial sutures (Gray, 2000)

Embryologically, the neurocranium develops from a small size, which facilitates vaginal birth, to a much larger adult size. To allow for brain growth and development, the overlaying cranial vault must expand in size. Cranial vault growth begins as increased cranial pressure rises from brain matter growth. The pressure on the cranial vault induces tensile forces at cranial sutures, causing separation of the bones juxtaposed bones, which is followed by compensatory

growth. Cranial sutures are considered essential growth centers of the skull. The method of growth that occurs at sutures is by appositional bone deposition. Therefore, in order for brain growth to occur, these sutures must contain osteo-potential cells (brain growth relies on appositional growth found at the sutures between cranial vault bones), and the neighboring bones must not fuse to one another (Gray, 2000). After cranial growth and development is completed, the four primary cranial sutures (metopic, coronal, sagittal, and lambdoid; Slater, 2008) will fuse. The metopic suture closes during the first year of life (Cunningham, 2007). The coronal, sagittal and lambdoid sutures close between the third and fifth year of life (Cunningham, 2007). Other cranial growth locations also close after growth has ceased; such as the closure of the spheno-occipital synchondrosis occurs between ages 15-20 (Powell, 1963; Cendekiawan. 2010); the spheno-ethmoidal synchondrosis closes early around 6 years of age; and the intersphenoid synchondrosis closes immediately after birth (Cendekiawan, 2010).

Nevertheless, premature fusion of cranial sutures during the developmental process has been seen in humans, rabbits, rats, and other species (Akita, 1994; Mooney, 1994; Gardner, 1998). Cranial suture fusion may occur due to a genetic or environmental reason (an insult originating from the environment of the fetus/child; such as drug, application of force, or behavior), or a combination of both. A common example of an environmental insult may be due to a mechanical condition, where reduced cranial pressure may lead to suture fusion. This is explained by neighboring bones approximating one another too closely and/or for too long a period of time that they may thereby fuse prematurely during fetal or adolescent growth and devolvement (Jacob, 2007). Future growth at that cranial suture will then cease.

Cranial sutures are a type of anatomic joint. In the body, all joints are derived of three basic types: **synarthrosis**, immovable; **amphiarthrosis**, slightly movable; **diathrosis**, freely

movable. The bony joints in the skull are mostly **synarthrotic**, and no appreciable movement occurs (syn, lack of; arthrosis, joint). Cranial sutures belong to the **synarthrotic** group, and are joint spaces of two bones that almost meet, except for a band of connective tissue or hyaline cartilage. Motion is not intended at these joints. Within the **synarthrotic** group are four variations: **sutura**, **schindylesis**, **gomphosis**, and **synchondrosis**. As evident by its word, **sutura** is Latin for suture, and **sutura** are only found in the skull. The connective tissue separating the two bones of a suture is thin fibrous tissue. And among sutures, there are further categories. **True sutures** are when margins of two bones interlock together by processes and indentations, and three variations occur: **sutura dentate**, **serrate**, and **limbosa**. The biparietal bone suture (also known as the sagittal suture) exhibits tooth-like projections and is called **sutura dentate.** The suture between the two frontal bones (metopic suture), shows small serrated teeth, like from a fine saw, and is called **sutura serrate**. Between the frontal and parietal bones (coronal suture), inter-lockings occur with a degree of overlap of the entire body of a bone, and is called **sutura limbosa**. **False Sutures** consist of roughed borders of two bones, and placed in opposition to one another, but lack the interlocking processes. Of this, there are two kinds: **sutura squamosa**, which is found between temporal and parietal bones and exhibits overlapping of the two bones; **sutura harmonia**, found between the two maxillae (and palatine bones) and demonstrates continuous rough surfaces. (Gray, 2000)

Other types of synarthrotic joints are **gomphosis**, **synchondrosis, and schindylesis joints. Gomphosis** articulations are found as the periodontal membrane connecting teeth to the alveolus. **Synchondrosis** articulations are where two adjoining bones meet with cartilage found between them: these articulations are considered important growth centers in the skull. **Schindylesis** articulations occur when thin laminae of bone insert perpendicularly into a

depression of another bone, such as the perpendicular plate of ethmoid or the vomer bone into the maxillae or rostrum of sphenoid bones (Gray, 2000)

Cranial sutures may allow for movement, in spite of being part of the synarthrotic group. Movement at cranial sutures, particularly fontanelles (a confluence of two or more sutures; Gray, 2000), is necessary during vaginal birth, where cranial bones may shift, and allowing passage of the baby. Fusion of a cranial suture will inhibit any movement at the joint. Therefore, the term synostosis (syn- no movement; ostosis- between boney segments) is used to describe a fused cranial suture; and the definition of craniosynostosis is a lack of movement between the two boney segments of a cranial suture (Gray, 2000; Kabbani, 2004; Delashaw, 1989).

Fusion of a cranial suture is a normal and physiologic result for all cranial sutures once normal and physiologic growth is complete. Once growth and development of the brain has ceased and reached maturity (brain growth plateaus at age 6, but development, such as myelination may continue well into 6^{th} decade of life; Benes, 1998), fusion occurs per the described mechanism as previously mentioned (tensile forces no longer occur at two boney segments of a suture, whereby separating them, as they approximate one another they naturally fuse). If fusion happens pathologically at a suture, prior to the brain reaching total growth, then the clinical consequences can range from being non-serious and undetected, to very serious with craniofacial, mental, ophthalmic, auditory, or maxillofacial deformities (Gray, 2000). Just as clinical signs and symptoms of craniosynostosis may vary, so may vary the causes of craniosynostosis. Causes may range from isolated cases involving a first generation point-mutation(s), to being part of a syndrome, or a combination of a variety of genetic and environmental conditions (Gray, 2000)

Classification systems of craniosynostosis have evolved over the years in an attempt to best describe the clinical differences of craniosynostosis. In the 19th century, Otto published a **Morphologic Nomenclature** that described and differentiated the variable appearances of head shape. At the beginning of the 20th century, a **Clinical Genetic Classification** system became more and more common, due to the occurrence of craniosynostosis in conjunction with genetic syndromes, such as Apert or Crouzon. Recently, in the 1990's, specific genes began to be isolated from genetic syndromes which display craniosynostosis (such as Pfeifer syndrome), and a separate system called **Molecular Genetic Classification** was developed (Wilkie, 1997; Cohen and MacLean, 2000). All three nomenclatures are commonly referred to today, and each has its role in understanding the diagnosis of craniosynostosis and etiology of its cause (Jones, 2002).

1.1.1 Morphologic Nomenclature

For centuries, and likely millennia, mankind has noted aberrant and dysmorphic head shapes. It is with little wonder that the first classification system for craniosynostosis would be based on **Morphologic Nomenclature** (see Table 1). To name a few: **Dolicocephaly** refers to elongated head appearance, and results from fusion of the sagittal suture. **Acrocephaly** is seen as a pointed head, and is a fusion of the coronal suture. Fusion of the metopic suture may be known as **trigonocephaly**. Many cases can involve unilateral or incomplete fusion of a suture, such as **plagiocephaly.** It is a fusion of a unilateral coronal suture, giving an asymmetric head appearance (Jones, 2002).

Table 1: Current Morphologic Nomenclature of craniosynostosis with phenotypic presentation and suture involved (Jones, 2002).

Term	Appearance	Affected Suture
Dolicocephaly	Long head	Sagittal suture
Scaphocephaly	Keel-shaped head	Sagittal suture
Acrocephaly	Pointed head	Coronal, Coronal/Lambdoid, or all sutures
Brachycephaly	Short head	Coronal suture
Oxycephaly	Tower-shaped head	Coronal/lambdoid or all sutures
Turricephally	Tower-shaped head	Coronal suture
Plagiocephaly	Asymmetric head	Unilateral coronal, unilateral lambdoid, or positional
Kleeblattschadel	Clover-leaf skull	Multiple but not all sutures
Craniofacial dysostosis	Midface deficiency	Craniosynostosis with involvement of cranial base sutures

1.1.2 Clinical Genetic Classification

In cases where craniosynostosis occurs with an array of other malformations, such as Apert's syndrome, an additional classification system is needed; hence the **Clinical Genetic Classification** system (see Table 2). In the 1980's, there began to be increase in documented cases of craniosynostosis, and evidence was found that nearly 80% of syndromes associated with craniosynostosis involved malformations in the limbs. The same genes that caused limb defects or syndactaly in syndromes were questioned at being responsible for craniosynostosis (Jones, 2002).

Table 2: Clinical Genetic Classification: Name of syndromic disorder with identified gene(s) (Jones, 2002; Johnson, 2011).

Diagnostic Category	Name of Disorder	Etiology
Isolated craniosynostosis	Morphologically described	Unknown, uterine constraint or FGFR3 or EFNA4 mutation
Syndromic craniosynostosis	Antley-Bixler syndrome	POR
	Apert syndrome	Usually one of two common mutations in FGFR2
	Bacre-Stevenson syndrome	Mutation in FGFR2 or FGFR3
	Baller-Gerold syndrome	Mutation in TWIST heterogenous
	Carpenter Syndrome	RAB23
	Crouzon Syndrome	Numerous different mutations in FGFR2
	Muenke syndrome	Mutation in FGFR3
	Pfeiffer syndrome	Mutation in FGFR1 or numerous mutations in FGFR2

1.1.3 Molecular Genetic Classification

In the 1990's, specific genes began to be identified in the syndromes that are related to craniosynostosis (Apert's, Crouzon's, Pfeifer, etc.). Mutations in FGFR1, 2 or 3 were seen in Pfeifer, Apert, Muenke, and other syndromes. Mutations in TWIST were seen Baller-Gerold or Saethre-Chotzen syndromes. Other genes have since been identified in other syndromes too. It is not clear how mutations in different genes and in different syndromes can cause

craniosynostosis, therefore a new classification for craniosynostosis was made; one based on **Molecular Genetic Classification**, see Table 3 (Jones, 2002)

Table 3: Identified genes of genetic syndromes that may involve craniosynostosis (Jones, 2002).

Gene	Mutation	Phenotype
FGFR1	755C through G	Pfeiffer syndrome (milder phenotype)
FGFR2	Multiple	Apert, Bacre-Stevenson, Crouzon, Jackson-Weiss, Pfeiffer syndrome (severe phenotype)
FGFR3	Multiple	Bacre-Stevenson, Crouzonodermoskeletal, Muenke syndrome
MSX2	Pro148His	Boston-type synostosis
TWIST	Multiple	Baller-Gerold, Saethre-Chotzen syndrome

Understanding the cause of craniosynostosis has been difficult due to, in part of, the small number of documented cases with the disease. Many cases of craniosynostosis may go un-noticed or unreported due to its milder forms or limitations to access of care. Furthermore, there may be carriers of a craniosynostotic gene who do not present with the phenotype. Not all cases report the same severity, indicating a variable phenotypic expression of craniosynostosis. Its incomplete penetrance of craniosynostosis is still unexplained.

1.2 EPIDEMIOLOGY OF CRANIOSYNOSTOSIS

1.2.1 Human Demographics and Epidemiology

Craniosynostosis occurs 1 in 2,000 to 2,500 live births worldwide and has no gender preference. The most commonly affected suture is the **sagittal suture**, occurring in about 40-55% of nonsyndromic cases. **Coronal synostosis** is the second most common type, comprising 20-25%, and **metopic synostosis** is third, comprising 5-15%. L**ambdoid synostosis** comprises only 0-5% of nonsyndromic cases. Nonsyndromic cases usually only affect one suture. When more than suture is affected, it is called complex craniosynostosis, and constitutes 5-15% of all cases. Generally they are related to syndromes (Slater, 2008)

The pathology of craniosynostosis has been well researched (Carmichel, 2008; Gardner, 1998; Honein, 2000; Kallen, 1999; Olshan, 1989; Jentink, 2010; Johnsonbaugh, 1978; Mulliken, 2004; Moloney, 1997; Passos-Bueno, 2008). Possible mechanisms are that the dura mater plays a critical role in the patency of sutures; others say the cause lies within the periosteum and connective tissue of the suture. Current research evaluates the gene/molecular interactions. Certain genes have already been isolated (FGFR1, 2, and 3, Tgf-br I and II, TWIST, and Msx1) that cause syndromic abnormalities, some being craniosynostosis. Nevertheless, the same affected gene is not present in all the syndromes that relate to craniosynostosis. Therefore, the likelihood that one gene causes craniosynostosis is remote. More likely, the cause of craniosynostosis is a combination of genes, mechanical/ biomechanical (either increased fetal pressure pre-birth, or decreased cranial pressure post birth), environmental or hormonal factors (Jacob, 2006 and 2007).

1.2.2 Diagnosis of Craniosynostosis

The diagnosis of craniosynostosis can only happen if a parent or physician suspects the clinical condition. It is made by from a physical examination, a review of family and personal medical history, and also by ultrasound or radiographic analysis. Usually diagnosis, treatment, and care of individuals with craniosynostosis, are performed in a craniofacial center, which usually are associated with major metropolitan hospitals.

A child's misshaped head is a common reason that brings the patient in for diagnosis of craniosynostosis. Nevertheless a malformed (dysmorphic) head is not the only symptom of craniosynostosis that worries parents and gets the attention of physicians. When a suture fuses (for whichever reason) but brain growth continues, there is an increase in intracranial pressure (ICP). Its symptoms can be quite severe (headaches, nausea, vomiting, lethargy, etc.). Children with craniosynostosis present with symptoms of ICP in 4-20% of cases. If more than one suture is involved in craniosynostosis, symptoms of ICP may be present in 62% of patients (Thompson, 2008).

Physical examination of a patient with craniosynostosis measures the head circumference, assesses for skull and limb deformities, and plots the child's growth curve. Skull deformities can be assessed from a superior view (bird's eye view), posterior view, and/or anterior view of the skull. Asymmetry is key to identify abnormal growth, including the eyes, ears, and nose in addition to overall head shape.

Computed axial tomography (CT) is the gold standard for radiographic analysis and diagnosis of craniosynostosis. Single suture synostosis can be identified with 2D radiographs, such as dorsoventral or lateral cephalogram radiographs. But CT scans are much more effective at visualizing the entire skull and identifying the scope of deformity (Medina, 2000).

Craniosynostosis can occur prior to birth, early on-set, or after birth delayed on-set (Reddy et al. 1990; Cohen et al. 1993). As previously mentioned, craniosynostosis occurs sporadically in the population and also in families. When craniosynostosis is repeated in a family pedigree, it is called familial craniosynostosis, and genes are likely the cause (Cohen et al. 1993). If a teratogen was exposed to the entire family, an environmental condition could theoretically cause familial craniosynostosis. No such cases have yet been documented. Since sporadic cases of craniosynostosis are very few, and their causes may differ extremely, it has been easier to study familial craniosynostosis, either related with a syndrome or not. Sporadic cases appear to be more biomechanically caused, while familial CS seems to be more genetic. Nevertheless, only 15% of familial cases have had genes identified with them.

Familial craniosynostosis is fairly common, and many reports show that environmental influences in these families may increase the penetrance and/or expression of craniosynostosis (Kosnik et al. 1975; Hunter and Rudd, 1976 and 1977; Cohen et al. 1993; Lajeunie et al. 1995, 1996, and 1998; Renier et al. 2000; Guimaraes-Ferreira et al. 2001). Familial craniosynostosis may be extremely varied amongst family members. This variable expression and phenotypic heterogeneity involves the number of sutures involved and timing of fusion. The variable expression of craniosynostosis in humans is similar to the variable expression of the rabbit colony used in this study (Kosnik et al. 1975; Lajeunie et al. 1995 and 1996; Moloney et al. 1997; Beaudet et al. 2001; Mooney et al. 1994a, 1994b, 1996, 1998, 2002).

An example of human phenotypic variability is when a parent has a mild form of a single synostotic suture, without mental impairment, while the child is severely affected with multiple suture early on-set synostosis and mental acuity impairment. Furthermore, a parent may only carry a gene related to craniosynostosis, but the children could show early on-set synostosis of

one or more suture, delayed on-set synostosis of one or more suture, or may not show any signs of synostosis (Kosnik et al. 1975; Hunter and Rudd, 1976 and 1977; Cohen et al. 1993; Lajeunie et al. 1995, 1996, and 1998; Renier et al. 2000; Guimaraes-Ferreira et al. 2001).

It's been suggested that the variable expression of fusion location and timing may be a result of either genetic or environmental factors. G**enetic factors** involve specific mutated ligands (as in the variability in different syndromes with FGFR2 mutations), Msx2 homeobox domain mutations, or an accumulation of modifier genes (i.e., inbreeding) (Muller et al. 1993; Jabs et al. 1993; Liu et al. 1995; Steinberger et al. 1996; Moloney et al. 1997; Reardon et al. 1997; Gripp et al. 1998; Renier et al. 2000; Cohen and MacLean, 2000; Cray, 2010). **Environmental factors** such as perinatal exposure (exogenous or endogenous) to teratogens or endocrine influences like androgens, insulin, or thyroid hormones (Friedman and Mills, 1969; Yip et al. 1980; Inouye et al. 1985; Alderman et al. 1994; Rothman et al. 1995; Gardner et al. 1998; Lajeunie et al. 2001; Duggan et al. 1970; Cohen and MacLean, 2000; Shashi and Hart, 2002). Maternal hyperthyroidism from autoimmune diseases such as Grave's disease in humans can result in early-onset craniosynostosis (Rasmussen, 2007). However, little is known about the interaction of these genes/genetic factors with environmental factors on incidence and penetrance of craniosynostosis.

1.2.3 Genetic Factors

Craniosynostosis has been related to genetic disorders. Fibroblast growth factor receptor 1, 2, or 3, Tgf-br I and II, TWIST, and Msx2 genes have been identified in some of the syndromes involving craniosynostosis (see Table 3). Fibroblast growth factors and receptors are found throughout connective tissue, and especially in cranial sutures. They regulate bone growth both

pre and post-natally. Mutations in these genes can cause gain of function, resulting in abnormal bone deposition and fusion. TWIST genes decrease the function of FGFR and indirectly control bone growth. Thus mutations in TWIST genes lead to loss of function. Craniosynostosis may be caused by small changes in the fine balance of bone regulation. These genes impact many tissues in the head, and other affected cranial structures are common. Apert, Crouzon, Peiffer, etc. all have other craniofacial abnormalities and dental disturbances. What remains unanswered is if all these disturbances are individually caused by genetic mutations, or if one deformity may result in another deformity (Wilkie, 1997).

1.2.4 Environmental Factors

Craniosynostosis may result from teratogen exposure (Friedman and Mills, 1969; Yip et al. 1980; Inouye et al. 1985; Alderman et al. 1994; Rothman et al. 1995; Gardner et al. 1998; Lajeunie et al. 2001), but more commonly results from a systemic metabolic disturbance (Duggan et al. 1970; Cohen and MacLean, 2000; Shashi and Hart, 2002). Epidemiological and case studies can describe relative associations, but isolating specific environmental factors is difficult due to the limited number of subjects in these studies (not achieving a strong statistical power) and the likelihood there are multiple environmental factors (inability to control for any one particular) (Shashi and Hart, 2002). The CDC ran an epidemiologic study that associated children born to mothers with thyroid hormone therapy to have increased odds ratio of 2.5 (Rasmussen et al. 2008). With that being said, there have been only a few environmental factors known to be related to craniosynostosis. Some of these are **vitamin D deficiency and rickets**, handful of **teratogens**, and **hyperthyroidism**.

1.2.5 Vitamin D Deficiency and Rickets

Rickets: is a condition related to a deficiency of **vitamin D** insufficiency to calcify growing bones in adolescents (Shashi and Hart, 2002). Deficiency of vitamin D may be due to lack of dietary intake, resistance of vitamin D, liver disease, chronic liver failure, and hypophosphatasia. Each of these forms of deficiencies has been linked to craniosynostosis (Coleman and Foote, 1954; Fraser, 1957; Reilly et al. 1964; McCarthy and Reid, 1980; Shashi and Hart, 2002). Infantile hypophosphatasia has frequently observed with craniosynostosis and ocular proptosis (Brenner et al. 1969). In genetic cases of rickets, craniosynostosis has been found as a secondary characteristic (Willlis and Beaty, 1997). Roy et al developed a mouse model with X-linked dominant hypophosphatasia that may be useful to study the development of craniosynostosis (Roy et al. 1981).

1.2.6 Teratogens

With regards to **teratogen** influence in craniosynostosis, evidence has mainly been limited to case reports. Nevertheless, there is evidence that maternal smoking has been linked to increased risk of craniosynostosis. Phenytoin exposure during pregnancy can lead to synosotosis of the sagittal and coronal sutures (Char et al. 1978). Metopic ridging has been observed from anticonvulsant treatment with valproate (Ardinger et al. 1988). Amine containing drugs (cyclophosphamide, alkylating agent in cancer chemotherapy) exposed to a fetus may also be linked to CS (Mutchinick et al. 1992; Enns et al. 1999).

1.2.7 Hyperthyroidism

Increased exposure to thyroid hormone has been linked to craniosynostosis. Thyroid hormones, thyroxine (T4) or tri-iodothyronine (T3), are produced in the thyroid gland (Walter, 2003). These tyrosine based hormones' primary role is to regulate metabolism (Walter, 2003). Thyroxin affects nearly all cells in the body (Dratman, 1996). Thyroid hormones have been found to increase metabolic rate of protein, fat and carbohydrates in all cells, affect synthesis of protein, regulate long bone growth and neuronal development, increase body's sensitivity to catecholamines, and promote vitamin metabolism (Dratman, 1996). Thyroid related conditions have been seen with either excess or lack of thyroid hormone production (Torre et al. 2008).

Thyroxine (T4) is produced 20 times more than T3, and is considered more stable due to its longer half-life than the 2-3 day half-life of T3 (Wiersinga, 2001). Thyroxine (T4) is converted to tri-iodothyronine (T3) by di-iodination of 4 molecular iodines to 3 (Simonides, 2008; Walter, 2003). Tri-iodothyronine is the most active form of thyroid hormones (Walter, 2003). Thyroxine (T4) acts as a reservoir for T3 (Wiersinga, 2001). Conversion of T3 is accomplished mainly within target cells (Walter, 2003). Tri-iodothyronine (T3) has a 10-fold greater affinity for thyroid receptors than T4 in cells (Samuels, 1974; Bianco, 2002).

Thyroid hormone has been found to affect the bone remodeling process. Bone remodeling is regulated by numerous calcitropic hormones (parathyroid hormones, sex steroids, thyroid hormones, etc.; Akita, 1996; Eriksen, 2010). The effect of thyroid hormones on bone remodeling differs at different stages of development (Akita, 1996; Eriksen, 2010; Mysliwiec, 2007c). In children, excess thyroid hormone increases skeletal bone formation, while in adults, hyperthyroidism causes bone loss due to increased bone turnover rate and increased bone resorption (Akita, 1996). In the presence of thyrotoxicosis, normal bone remodeling process

decreases from 200 days to only 100 days (Eriksen, 2010). In elderly women, osteoporosis may be related to increased levels of interleukin-6 (IL-6) and thyrotoxicosis (Mysliwiec, 2007c). Interleukin-6 (IL-6) appeared to play a crucial role in thyrotoxicosis-related disturbances, mostly by inhibition of bone formation, in an animal study of mice (Mysliwiec, 2007a). Osteoclastic activity decreased in rats with hypothyroid treatment (Mysliwiec, 2007b). Decreased bone density has also been seen in postmenopausal women who were treated with thyroxine (Guo, 1997).

Hyperthyroidism, whether congenital (as in auto-immune Graves' disease) or as a consequence of thyroid replacement hormone therapy, has been associated with craniosynostosis in numerous reports throughout the pediatric and endocrinology literature (Robinson et al. 1969; Duggan et al. 1970; Menking et al. 1972; Riggs et al. 1972; Penfold and Simpson, 1975; Hollingsworth and Mabry, 1976; Johnsonbaugh et al. 1978; Daneman et al. 1980; Floret et al. 1980; Cove and Johnson, 1985; Leonard et al. 1987; Krude et al. 1997; Segni et al. 1999; Zimmerman, 1999; Bieberman et al. 2000; Shashi and Hart, 2002). Children, born from mothers with hyperthyroidism (hyperthyroid state due to exogenous treatment for hypothyroidism, or endogenous over production of thyroid hormones), have been observed with craniosynostosis (Leonard et al. 1987).

The mechanism of hyperthyroid induced craniosynostosis is not entirely clear. While generalized skeletal maturation is a common finding in fetal, infant, and childhood hyperthyroidism (Schlesinger and Fisher, 1951; Riggs et al. 1972; Johnsonbaugh et al. 1978; Cove and Johnston, 1985; Schwab et al. 1996), it is believed that precocious bone maturation itself is not the cause of the craniosynostosis (Johnsonbaugh et al. 1978; Cohen and MacLean, 2000). For example, Johnsonbaugh et al. (1978) noted that in cases of congenital adrenal

hyperplasia, bone age is typically advanced, but no association with craniosynostosis exists. This suggests that although thyroid hormone (TH) may increase skeletal maturation, its pathway to do so is different than the androgens or mineralocorticoids involved in adrenal hyperplasia. Thyroid hormone may act locally to stimulate sutural ossification through one or more designated biochemical pathways, or perhaps centrally through yet undiscovered pathways.

Akita studied fusing sutures in a hyperthyroid rat model (Akita et al 1994, 1996). Sixty (60) Wistar rats, n=30 in a treatment group of tri-iodothyronine (T3), received 0.1 microgram/gram of T3 per body weight per day starting at 10 days of age. Control group, n=30, were used to compare. The rats were sacrificed at 15, 30, and 60 days. Morphologic measurements found that lambda-asterion and pterion-bregma distances were significantly decreased. Histologic findings showed fluorescent labeling without interruption along the sutures, indicating narrowing of the sagittal suture with continuous bone formation. Tartrate resistant acid phosphatase staining, in the T3 treatment group, revealed little osteoclastic activity in the sagittal suture. Local IGF-1 was markedly increased in the suture margins. Local IGF-1 previously has been found to play a critical role in suture formation of in vivo bone models, being the most abundant growth factor in osteoblasts (Krieger et al. 1988; Wolf et al. 1989; Lakatos et al. 1993; 2000; Thaller et al. 1993a, 1993b, and 1996; Varga et al. 1994; Klaushofer et al. 1995; Huang et al. 2000; Conover and Rosen, 2002; Stern, 2002). Akita concluded that excess administration of thyroid hormone enhanced the cranial suture closure, increased local IGF-1, and that local IGF-I played an important role in the sutural closure (Akita et al. 1996). Further studies contribute to understanding the general osteogenic role of both TH and local IGF-1 (Canalis, 1980; Rizzoli et al. 1986; Canalis et al. 1988; Krieger et al. 1988; Wolf et al. 1989; Lakatos et al. 1993 and 2000; Thaller et al. 1993a and 1993c; Varga et al. 1994; Klaushofer et al.

1995; Linkhart et al. 1996; Wakisaka et al. 1998; Huang et al. 2000; Rizos et al. 2001; Conover and Rosen, 2002; Stern, 2002).

1.3 AIM OF STUDY

The aim of this project was to identify the environmental influence of post-natal administration of tri-iodothyronine (T3) in rabbits that demonstrate familial delayed onset craniosynostosis when compared in-colony normal control rabbits. The hypothesis was tested in a well-established rabbit model of familial craniosynostosis with variable expression (Mooney et al. 1994a, 1994b, 1996, 1998, 2002). This rabbit model, similar to humans, demonstrates autosomal dominant transmission with incomplete penetrance (Mooney et al. 1996), and a broad range of phenotypic expression that includes: phenotypically normal animals that carry the mutation, unilaterally affected animals with postnatal or delayed-onset synostosis, animals presenting with complete bilateral fusion with prenatal or early-onset, or animals with severe synostosis that do not survive (Mooney et al. 1998).

These rabbits possess a broad spectrum phenotype and provide a unique opportunity for investigating the relationship between circulating thyroid hormones and suture pathology. In the presence of excess tri-iodothyronine (T3), the pattern of craniosynostotic progression in affected and unaffected rabbits were altered, such that changes in the timing and severity of sutural hyperostosis were followed.

1.4 PURPOSE OF PRESENT INVESTIGATION

Since there is evidence that pre- and post-natal exposure to maternal hyperthyroidism can affect suture development and patency in normal individuals (Johnsonbaugh et al. 1978; Riggs et al. 1972; Menking et al. 1972; Hollingsworth et al. 1976; Daneman et al. 1980), it was hypothesized that an interaction of postnatal treatment of exogenous tri-iodothyronine (T3) and a genetic propensity for synostosis would accelerate suture fusion and result in more severe phenotypes in individuals with a synostotic phenotype compared to controls.

In particular, it was hypothesized that a 17 day long, postnatal exposure to tri-iodothyronine (T3) in 25 day old rabbits that have delayed-onset synostosis (typical fusion and plateau of cranial growth is about 42 days of age for rabbits used in this study) would result in more decreased coronal suture growth than treated or untreated in-colony normal control rabbits and untreated delayed on-set coronal suture synostotic rabbits, pointing towards a gene/environment interaction of T3 with affected genes found in our colony of rabbits.

1.5 CLINCAL SIGNIFICANCE

Certainly, humans exhibit a natural range of variation regarding thyroid hormone production and/or receptor affinity, and sub-clinical cases of hyperthyroidism exist (Refetoff et al. 2001). In terms of treatment, individuals with sub-clinical or clinical hyperthyroidism may be at greater risk for craniosynostosis. Controlling thyroid hormone levels in these individuals may mitigate the risk of post-operative re-synostosis may follow suturectomy (Moss, 1959; Norwood et al. 1974; Marchac and Renier, 1982, Marsh and Vannier, 1985; Ousterhout and Vargervic, 1987;

Persing et al. 1989; Hassler and Zentner, 1990; Fatah et al. 1992; Drake et al. 1993; Hudgins et al. 1998; Jane and Persing, 1986; Mooney et al. 2001).

1.6 HYPOTHESIS

Postnatal exogenous hyperthyroid exposure would accelerate coronal suture fusion and affect subsequent calvarial growth more severely in rabbits with familial delayed-onset synostosis than in-colony control rabbits.

2.0 MATERIALS AND METHODS

Sixty five rabbits were utilized in the present study. A 2 x 3 x 3 (phenotype x treatment x age) design will be employed with 10 rabbits per group. The phenotype variable included), phenotypically normal in-colony rabbits (n=24), and rabbits with delayed onset coronal suture synostosis (n=41). The treatment groups included untreated controls, sham or vehicle treated controls, and rabbits treated with tri-iodothyronine (T3). Vehicle control treatments groups received buffered saline every three days from 25 days of age to 42. Surgical sham control groups only received amalgam markers with no further treatment. Thyroid hormone treatment group received 0.2mg/kg dose of tri-iodothyronine (T3) every 3 days beginning at 25 days of age to 42. The age variable included radiographic data collected on each rabbit at 10, 25, and 42 days of age.

2.1 ACQUISITION OF SAMPLE

A total of 65 New Zealand white rabbits (*Oryctolagus cuniculus*) were utilized for this study. Following a standardized breeding protocol (Losken et al. 1993; Mooney et al. 1994b) 41 rabbits with delayed-onset craniosynostosis were obtained from an existing breeding colony with variably expressed familial, non-syndromic coronal suture synostosis. Twenty four (24) normal in-colony rabbits were obtained from the same existing breeding colony, but without coronal

suture synostosis. All rabbits were housed and maintained in the Physical Anthropology Laboratory and Vivarium (University of Pittsburgh, Department of Anthropology). All rabbits were fed a standard diet of rabbit chow and water for the duration of the experiment. Full IACUC approval was obtained for this study.

2.2 DETERMINING AFFECTION STATUS AND IMPLANTING AMALGAM MARKERS

At 10 days, radiopaque suture markers were implanted to monitor suture growth. First, rabbits were anesthetized with an intramuscular (IM) injection of a solution comprised of 91% Ketaset (ketamine hydrochloride, 100mg/ml) and 9% Rompun (xylazine hydrochloride, 20mg/ml) at a dose of 0.59ml/kg body weight. Following anesthetization, hair was removed from the scalp and prepared with betadine and a midline incision was made with a surgical knife in the skin overlying the calvaria. The skin was then undermined and reflected to facilitate visual inspection of the sutures. At this point, an initial visual diagnosis was attempted for all rabbits based on synostotic progression. A 0.4mm dental burr was used to make six holes in the periosteum and bone. Three sets of holes were made 2mm lateral to the mid-sagittal plane on the animal's left side: 2mm anterior and posterior to the coronal, frontonasal and anterior lambdoidal sutures. Each hole was then packed with silver amalgam to serve as radiopaque markers. Following marker implantation, the skin was closed with 4-0 resorbable vicryl sutures and the rabbits were administered antibiotics (Baytril—Bayer Health Care LLC, from Med Vet International) through IM injection in order to prevent post-operative infections. All rabbits were monitored closely to assure full recovery.

The delayed-onset rabbit model presented with gross sutural abnormalities by 25 days of age (Mooney et al., 1994b). Following the initial attempt at 10 days to visually diagnose the rabbits, suture marker separation from radiographs (see Data Acquisition below) was used to confirm initial observations and diagnose DOS. By plotting sutural growth against somatic growth curves, it was possible to diagnose rabbits as having slow growing or normal sutures by 25 days of age. Rabbits that had less than 2.2mm of bilateral coronal marker separation were given the diagnosis of delayed on-set craniosynostosis. Those that had more than 2.2mm bilaterally were diagnosed as normal rabbits.

2.3 DEVELOPING THE EXPERIMENTAL MODEL

Following diagnostic confirmation at 25 days of age, rabbits within each of the 2 phenotypes (in-colony normal and delayed onset synostosis rabbits) were also randomly assigned to one of the three treatment groups. These three groups included: 1) no-treatment controls, 2) vehicle injection control group (sham), and 3) administered tri-iodothyronine (T3). For 17 days, beginning at 25 days of age, rabbits in the treatment groups received subcutaneous injections every three days of tri-iodothyronine (half-life 2-3 days, Wiersinga, 2001) dissolved in water/ethanol solution, buffered with sodium hydroxide, at a dose of 200 μg/kg body weight similar to therapeutic hormone replacement dose for hypothyroidism (Tremblay et al. 1977; Banerjee, 1983; Chizzonite et al. 1984; Sadiq et al. 1985; Saeki et al. 1987; Seiden et al. 1989; Goto et al. 1990; Szymanska et al. 1991; Boerth and Artman, 1996; Jiang et al. 2000; Ozdemirci et al. 2001). Rabbits in the vehicle injection control (sham) group likewise received treatments for 17 days beginning at day 25, and every third day through 42 days. These injections only

contained the saline solution without tri-iodothyronine. Serum T3 and T4 levels were taken at 42 days from the marginal ear vein with a 25 gauge butterfly needle, and analyzed by enzyme immunoassay (AHDC Endocrinology Laboratory, Cornell University, Ithaca, NY). All treatment ended when the rabbits reached 42 days of age. The treatment period, between 25 and 42 days, was chosen because it is characterized by rapid somatic and craniofacial growth. Data collection stopped at 42 days of age due to craniofacial growth plateau at 42 days of age for New Zealand white rabbits.

2.4 DATA ANALYSIS

2.4.1 Cephalometric Data Analysis

For each rabbit, serial body weights were recorded and both lateral and dorsoventral radiographs of the calvaria were carried out at 10, 25, and 42 days of age. During the dorsoventral radiograph the forepaw of each animal was positioned for assessment of somatic skeletal growth. Following a standardized protocol (Mooney et al., 1993), each rabbit was tranquilized with an IM injection of Ketaset (ketamine hydrochloride, 100mg/ml) at a dose of 10mg/kg body weight. Following tranquilization, the heads were positioned in a specially designed cephalostat. All radiographs were carried out on a Phillips Oralix 70 x-ray unit at an exposure of 50kV, 7mA for 0.17 to 0.50 seconds, with a constant tube to cassette distance of 152cm (Phillips and Company, Washington, DC)

Once the radiographs were acquired, each was viewed after being scanned digitally using a Kodak Point of Care CR 120 and stored as image files on a Gateway 2000 PC (Eastman Kodak

Company, Rochester, NY). Using the image-measuring program Scanpro (Jandel Scientific), amalgam marker separation (a measure of growth at the suture site) at the coronal, frontonasal and lambdoidal sutures was quantified.

Cephalometric landmarks (13; see table 4, figure 1) used in previous studies on lateral cephalometric (Mooney et al., 1994a, 1994b), were identified by one operator for all radiographs from all time points (see table). Results were obtained from linear measurement of coronal suture marker separation (figure 2), total craniofacial length (figure 3), cranial vault length (figure 4), cranial vault height, and cranial vault shape index (figure 5). Angular measurements obtained were cranial base angle (figure 6) and palatal angle (figure 7). Other results were reported for blood serum levels of T3 and T4 and body weight.

Table 4: Lateral cephalometric landmarks used in the study.

Landmark	Abbreviation	Definition
Opisthion	OP	Inferior tip of the occipital bone at the foramen magnum.
Maximum occipital point	MOP	Most posterior projecting point on the occipital bone.
Anterior lambdoid suture	ALS	Point demarcating the center of the anterior lambdoid suture between the osteogenic fronts.
Coronal suture	CS	Point demarcating the center of the anterior coronal suture between the osteogenic fronts.
Frontonasal suture	FNS	Point demarcating the center of the anterior frontonasal suture between the osteogenic fronts.
Rhinion	RH	Most anterior point on the nasal bone.
Prosthion	PR	Point where the alveolar bone of the maxilla meets the anterior surface of the upper incisor.
Upper molar point	UMP	Point where the alveolar bone of the maxilla meets the anterior surface of the first molar.

Basion	BA	The posterior tip of the basiocciput at the foramen magnum.
Spheno-occipital synchondrosis	SOS	Point demarcating the center of the articulation between the sphenoid and occipital bones on the endocranial surface.
Optic foramen	OF	Anatomical center of the optic foramen.
Pre-spheno-ethmoidal synchondrosis	PSES	Point demarcating the center of the articulation between the presphenoid (sphenoid) and ethmoid bones on the endocranial surface
Fronto-ethmoidale	FE	Point where the cribriform plate of the ethmoid articulates with the frontal bone on the endocranial surface

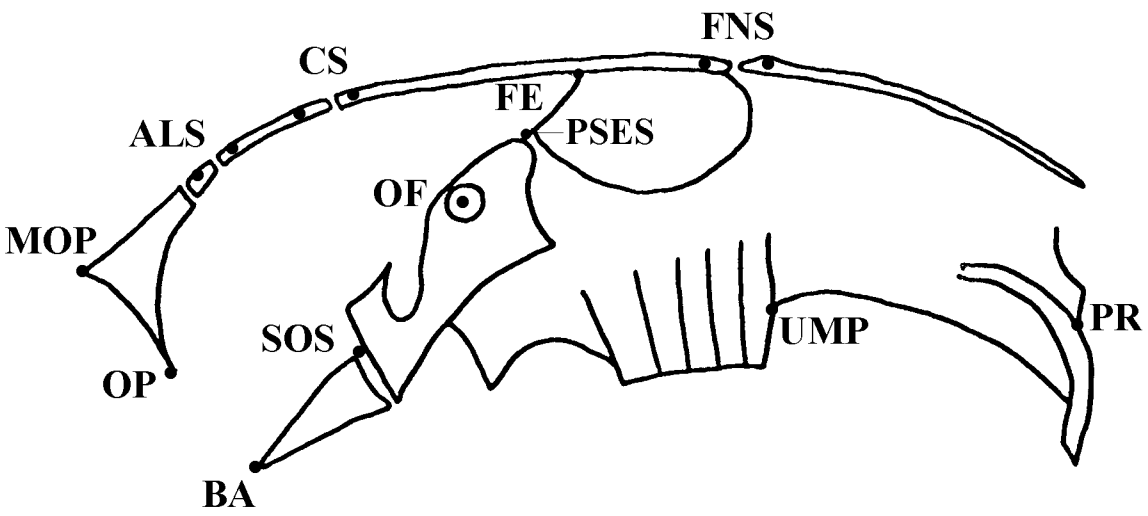

Figure 1: Traced lateral cephalogram with all 13 anatomic landmarks.

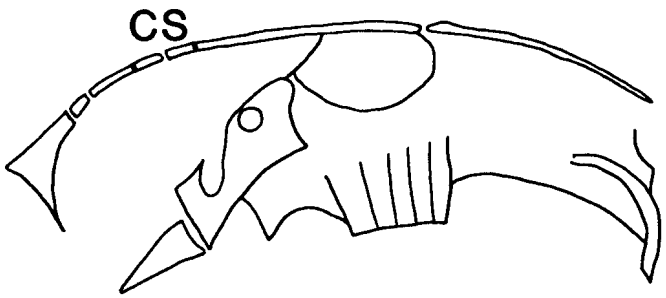

Figure 2: Coronal suture (CS) on traced lateral cephalogram.

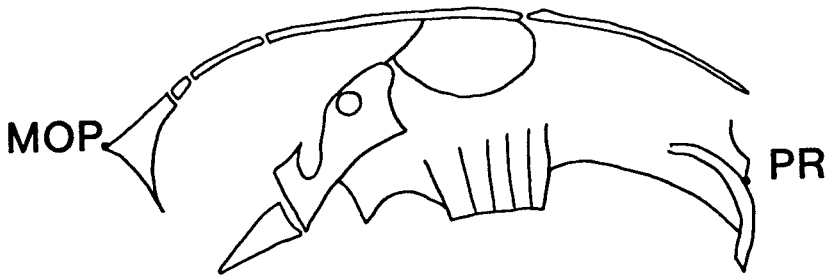

Figure 3: Total craniofacial length identified by maximum occipital point (MOP) and prosthion (PR).

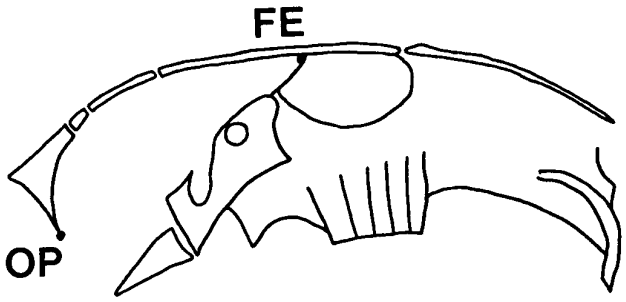

Figure 4: Total cranial vault length made by opisthion (OP) and fronto-ethmodale (FE).

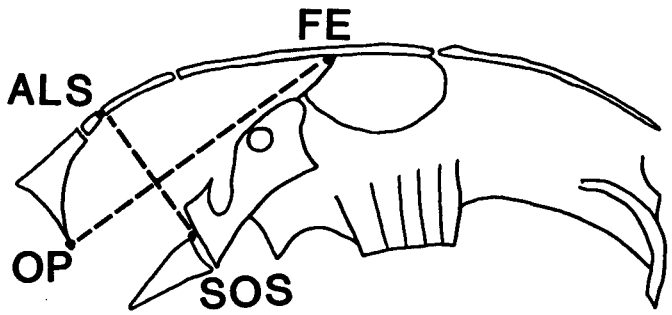

Figure 5: Cranial Vault Shape Index made from intersecting lines of anterior lambdoid suture (ALS) and spheno-occipital suture (SOS) with opisthion (OP) and fronto-ethmodale (FE).

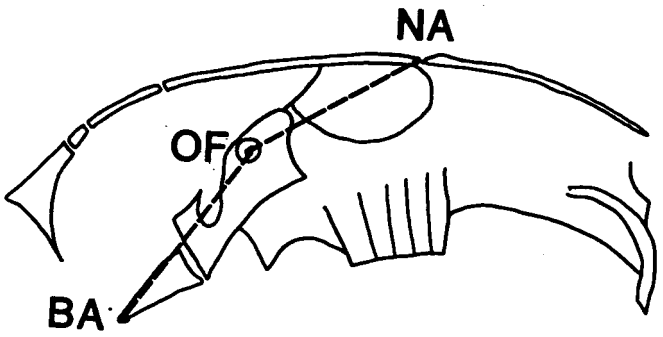

Figure 6: Cranial base angle from basion (BA), optic foramen (OF) and nasion (NA).

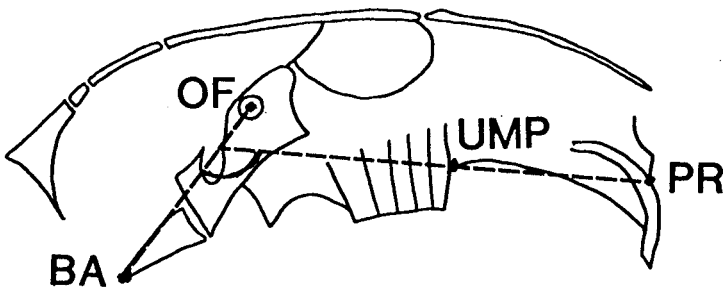

Figure 7: Palatal angle made by intersecting lines of basion (BA) and optic foreman (OF) with upper molar point (UMP) and prosthion (PR).

To assess intra-observer reliability, the entire set of landmarks was collected twice by a single rater from the lateral and dorsovental images of 13 animals. At least 48 hours separated the first and second landmarking sessions. The coordinate locations associated with each landmark (x-axis, y-axis) were saved and compared statistically across the two sessions by calculating intraclass correlation coefficients (ICCs: two-way mixed model with absolute agreement). This approach allows for reliability to be assessed for each landmark, in each axis separately. The average ICC across all landmarks in both axes was 0.999 (range: 0.989 – 1.000), indicating that observer-related measurement error was extremely low for all landmarks in all axes.

2.4.2 Morphometric Data Analysis

Differences in all linear dimensions and mean measurements were assessed both within and across groups with a two-way ANOVA at each age group. Post-hoc, pair wise comparisons were carried out with an LSD test for multiple comparisons

3.0 RESULTS

A total of 24 in-colony normal rabbits were included in this study as well as 41 delayed onset rabbits (See Table 5). The wild-type rabbits first enrolled in the study did not provide usable data and were too few in number to include in the results. Results for the delayed onset and in-colony normal rabbits are as follows:

Table 5: Number of rabbis in each group.

	Control	Vehicle	T3
ICN	8	10	6
DOS	14	14	13

3.1 THYROID HORMONE BLOOD LEVELS

Results of blood serum levels for tri-iodothyronine (T3) and thyroxine (T4) are represented in units of ng/ml and ug/ml respectively and are presented in Figures 8 and 9. Results show that mean T3 levels were significantly elevated in the delayed on-set and in-colony normal rabbits treated with T3 when compared to the control no-treatment or vehicle-control groups ($F = 5.96$, $p<0.005$, see tables 6 and 7, and figure 8). There was no statistical difference between levels of T3 between the treated groups ($F = 0.31$; p NS). Furthermore, there were no statistical differences in the control groups of either the delayed on-set or in-colony normal rabbits, meaning the untreated groups were not different from the colony as a whole ($F + 0.003$; p NS).

Table 6: Means of tri-iodothyronine (T3) blood levels in rabbits at 42 days of age.

		42 Days	
		Mean	SD
In-Colony Normal	Control	1.040	0.303
	Vehicle	1.153	0.207
	Surgery	2.273	2.188
Delayed Onset	Control	0.983	0.297
	Vehicle	1.140	0.177
	Surgery	2.827	2.690

Table 7: ANOVA statistics for tri-iodothyronine (T3) blood levels in rabbits at 42 days of age.

	42 Days	
	F Value	P Value
Phenotype	1.702	0.198
Treatment	4.534	0.015
Phenotype*Treatment	2.354	0.105

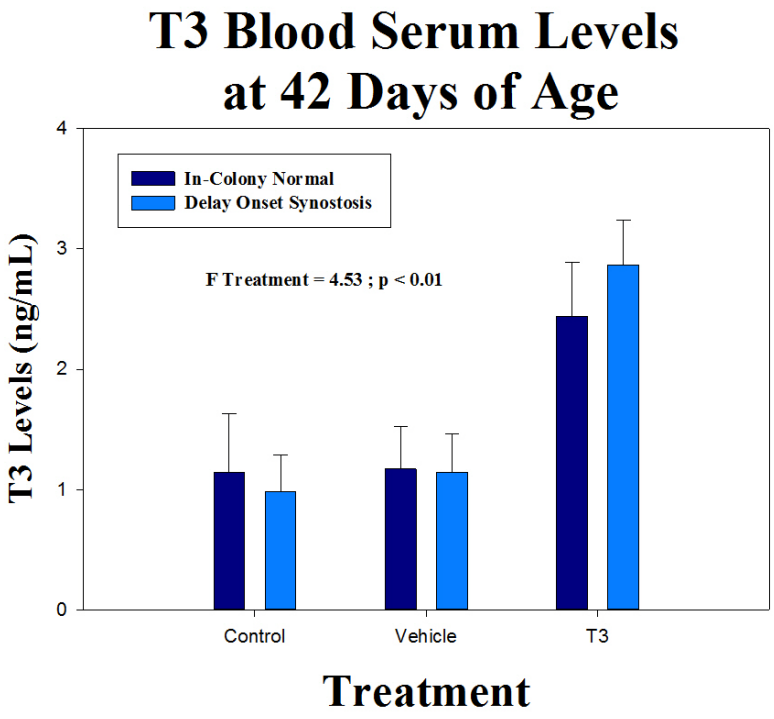

Figure 8: T3 levels significantly increased in treated rabbits.

Mean levels of T4 were significantly lowered in both treatment groups (delayed on-set rabbits or in-colony normal rabbits) ($F = 41.07$; $p<0.000$, see tables 8 and 9, and Figure 9). There were no statistical differences of T4 levels between the two treated groups ($F = 0.34$; p NS). There were no varying levels of T4 between the control groups of either phenotype, delayed on-set or in-colony normal ($F = 0.42$; p NS).

Table 8: Means of thyroxine (T4) blood levels in rabbits at 42 days of age.

		42 Days	
		Mean	SD
In-Colony Normal	Control	1.825	0.637
	Vehicle	1.984	0.473
	Surgery	0.064	0.931
Delayed Onset	Control	1.513	0.646
	Vehicle	1.971	0.885
	Surgery	0.047	0.008

Table 9: ANOVA statistics for T4 blood levels

	42 Days	
	F Value	P Value
Phenotype	0.420	0.520
Treatment	41.063	0.000
Phenotype*Treatment	0.340	0.714

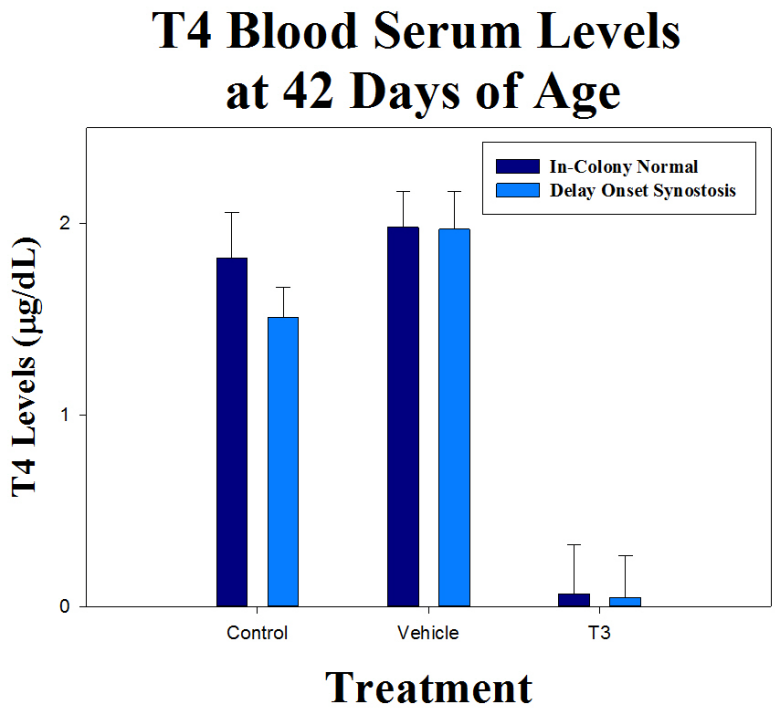

Figure 9: T4 levels significantly decreased in treated rabbits.

3.2 CORONAL SUTURE MARKER SEPARATION

Figure 4 shows the coronal suture marker separation means by phenotype and control group. Visual separation of the treated groups from their control groups is apparent, indicating a trend towards effect. Coronal suture marker separation was measured from amalgam marker on either side of the suture (see Figures 10 and 11). Coronal suture marker separation between both phenotypes was significantly different, with the delayed on-set rabbits showing statistically significantly ($F = 32.81$; $p<0.000$; see Tables 10 and 11) less coronal suture growth than the in-colony normal rabbits. Rabbits treated with T3 showed less coronal suture marker separation from 25 to 42 days of age however, this was not statistically different compared to controls ($F = 2.07$; p NS) nor was there a statistically significant difference among any one phenotype's three sub-treatment groups ($F = 0.65$; p NS).

Table 10: Means of coronal marker suture separation at 25 and 42 days of age.

		25 Days		42 Days	
		Mean	SD	Mean	SD
In-Colony Normal	Control	2.650	0.24	4.044	0.44
	Vehicle	2.655	0.61	4.325	0.70
	Surgery	2.617	0.24	3.650	0.27
Delayed Onset	Control	1.643	0.58	2.971	0.87
	Vehicle	1.775	0.40	3.135	0.73
	Surgery	1.892	0.35	2.941	0.39

Table 11: ANOVA statistics for coronal suture marker separation at 25 and 42 days of age.

	25 Days		42 Days	
	F Value	P Value	F Value	P Value
Phenotype	54.569	0.000	32.808	0.000
Treatment	0.273	0.762	2.067	0.136
Phenotype*Treatment	0.441	0.646	0.648	0.527

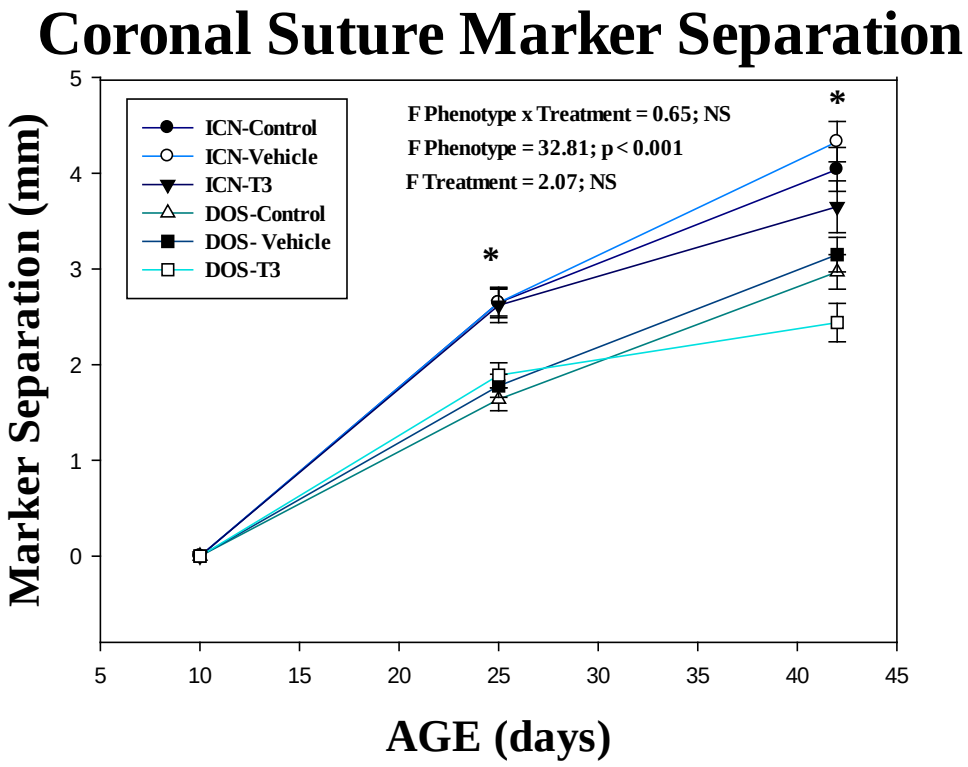

Figure 10: Delayed on-set rabbits showed significantly decreased coronal suture marker separation.

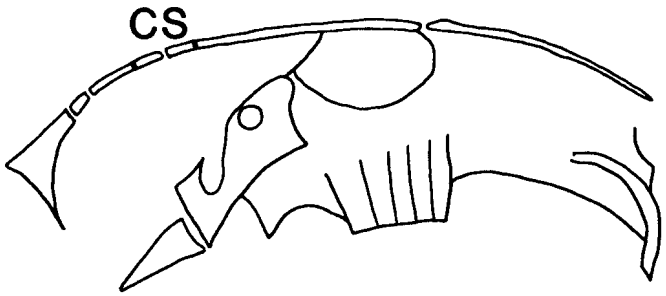

Figure 11: Coronal suture (CS) on traced lateral cephalogram.

3.3 BODY WEIGHT

Figure 5 shows mean changes in body weight by phenotype and control group. There were no statistical differences in body weight of any rabbits in any of the groups at age of 25 days (see Table 12 and 13). At 42 days of age, both treatment groups of T3 show markedly (see Figure 12) reduced body weights of the rabbits when compared to the control rabbits in control groups ($F = 6.91$; $p<0.002$). There were no statistical differences in rabbit body weights between control groups amongst the phenotype groups, nor between the two treatment groups in either phenotype group.

Table 12: Mean body weights at 25 and 42 days of age.

		25 Days		42 Days	
		Mean	SD	Mean	SD
In-Colony Normal	Control	0.340	0.057	0.984	0.082
	Vehicle	0.335	0.057	0.954	0.074
	Surgery	0.310	0.081	0.733	0.095
Delayed Onset	Control	0.418	0.041	0.862	0.062
	Vehicle	0.388	0.036	0.966	0.065
	Surgery	0.378	0.047	0.655	0.065

Table 13: ANOVA statistics for body weights at 25 and 42 days of age.

	25 Days		42 Days	
	F Value	P Value	F Value	P Value
Phenotype	1.086	0.343	1.060	0.308
Treatment	0.273	0.762	6.910*	0.002*
Phenotype*Treatment	0.287	0.885	0.483	0.632

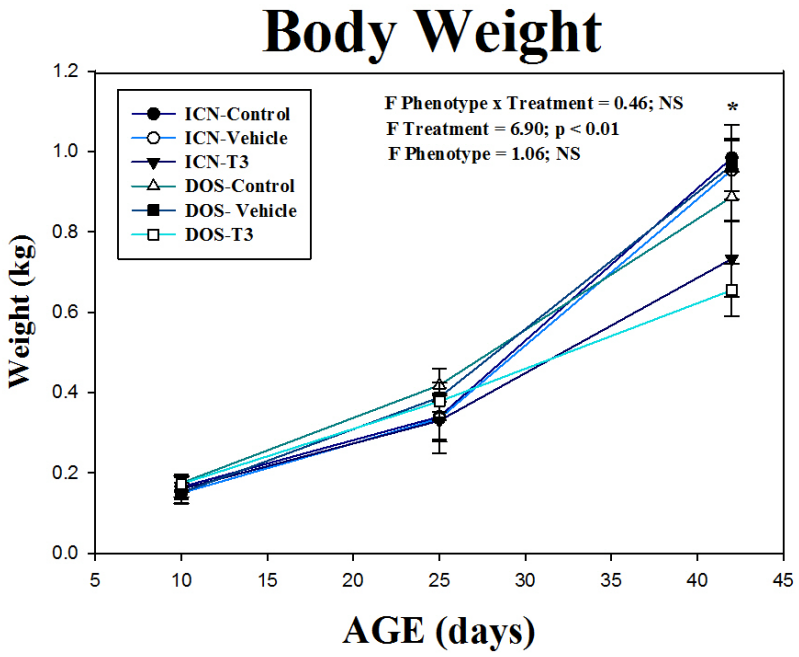

Figure 12: Treated rabbits with T3 show decreased body weights.

3.4 LATERAL CEPHALOGRAM VISUAL DIFFERENCES

Figure 13 represents six lateral cephalograms of in-colony normal rabbits and delayed on-set rabbits (DOS). A blue arrow visualizes the coronal suture, and coronal suture in the Normal, No Treatment rabbit is visually wider than the treatment (T3) rabbits. A red arrow demonstrates a slope from the frontonasal prominence towards the cranial vault. This visually demonstrates a different cranial vault shape in treated and delayed on-set rabbits.

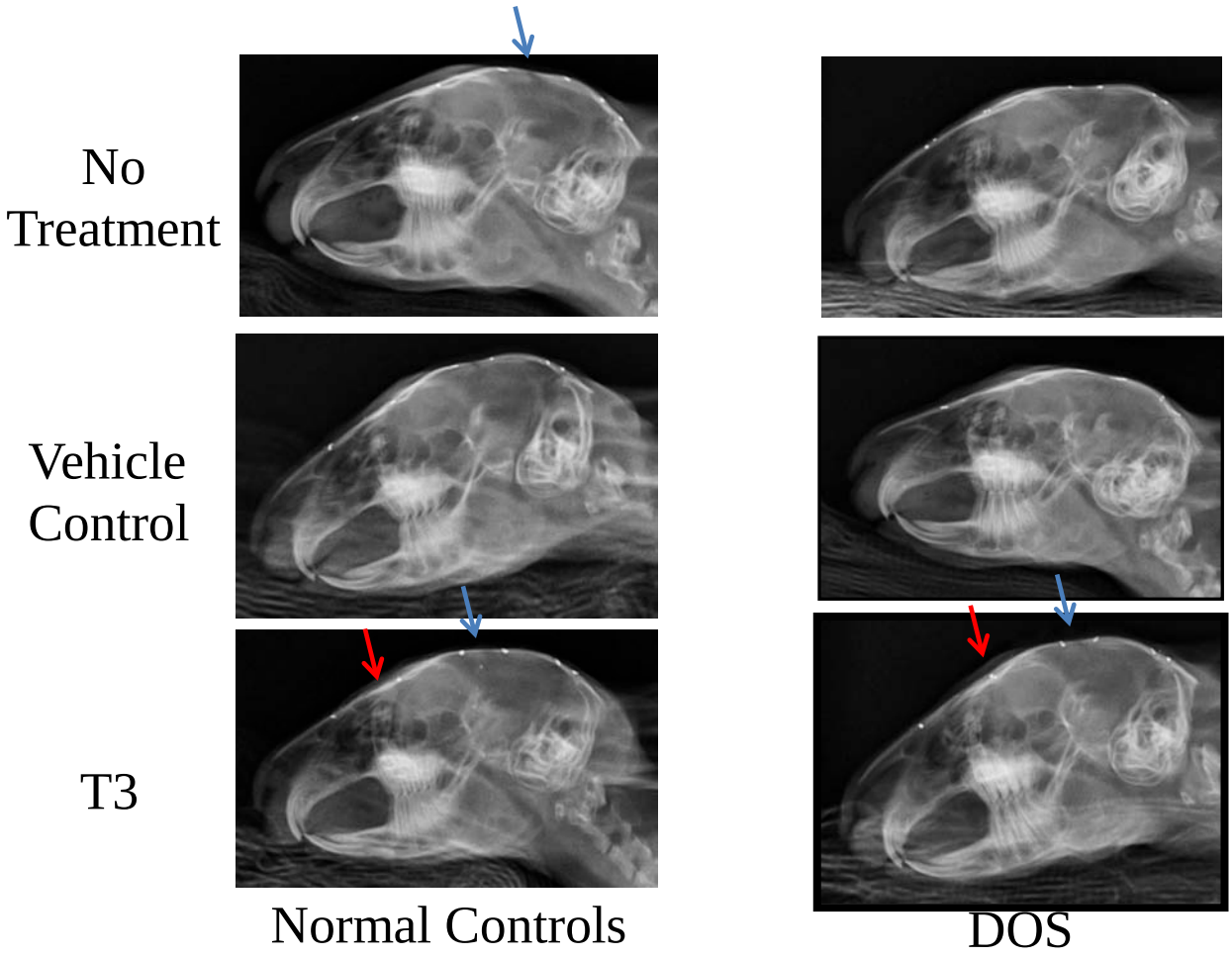

Figure 13: Lateral cephalograms for delayed on-set (DOS) and in-colony normal (ICN) rabbits. Blue arrows indicate coronal Sutures. Red arrows indicate taller cranium compared to frontonasal process.

3.5 TOTAL CRANIOFACIAL LENGTH

Figure 14 shows mean changes of craniofacial length by phenotype and control group. Measurements of the total craniofacial length revealed no statistical differences between the phenotype groups, treatment groups, or the phenotype-treatment groups at time points 10, 25, 42 days. See Tables 14 and 15, and Figure 14 and 15.

Table 14: Mean Total Craniofacial Length's at 10, 25, and 42 days of age.

		10 Days		25 Days		42 Days	
		Mean	SD	Mean	SD	Mean	SD
In-Colony Normal	Control	52.53	3.26	66.66	6.28	81.58	4.64
	Vehicle	50.22	4.06	66.17	3.37	81.12	3.63
	Surgery	52.82	2.38	67.75	4.49	80.17	5.35
Delayed Onset	Control	52.01	2.98	65.71	1.99	80.42	2.57
	Vehicle	51.94	3.71	66.76	3.86	81.52	3.59
	Surgery	51.87	2.61	66.09	3.51	79.26	3.62

Table 15: ANOVA statistics for total craniofacial length at 10, 25 and 42 days of age.

	10 Days		25 Days		42 Days	
	F Value	P Value	F Value	P Value	F Value	P Value
Phenotype	0.006	0.939	0.317	0.576	0.216	0.645
Treatment	0.768	0.470	0.109	0.897	0.670	0.518
Phenotype*Treatment	0.769	0.470	0.364	0.697	0.195	0.824

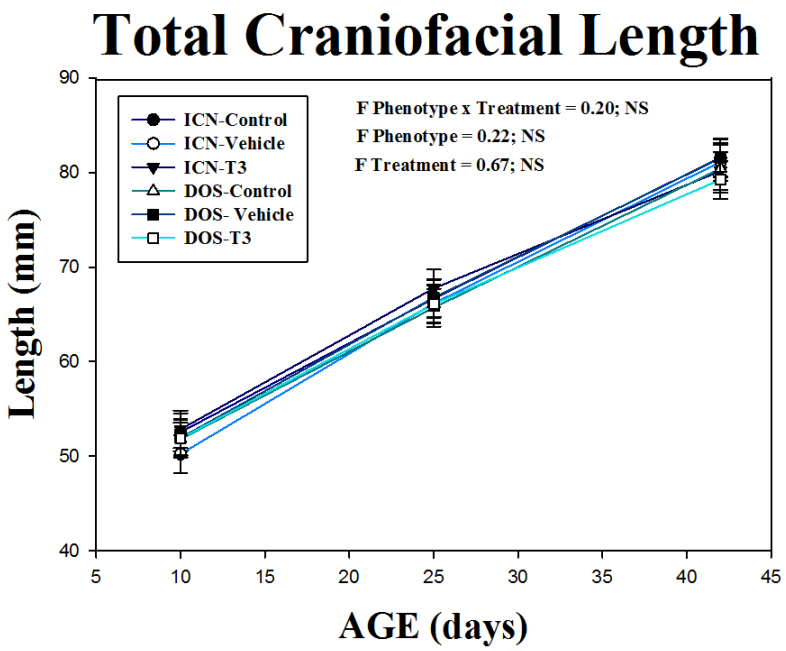

Figure 14: No significant difference in craniofacial lengths amongst the groups.

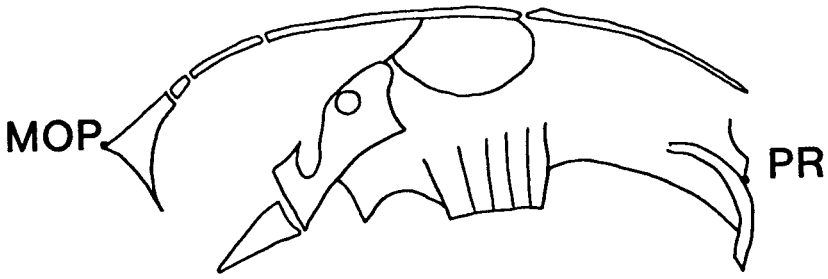

Figure 15: Total craniofacial length identified by maximum occipital point (MOP) and prosthion (PR).

3.6 CRANIAL VAULT HEIGHT

Measurements for cranial vault height were not significant for phenotype or treatment group at 10, 25 or 42 days of age. See Tables 16 and 17.

Table 16: Mean values for cranial vault height at days 10, 25, and 42 days of age.

		10 Days		25 Days		42 Days	
		Mean	SD	Mean	SD	Mean	SD
In-Colony Normal	Control	19.26	0.62	22.13	1.00	24.79	0.66
	Vehicle	18.71	1.03	21.78	0.84	23.56	0.70
	Surgery	19.40	0.86	22.11	1.25	23.36	1.79
Delayed Onset	Control	19.05	1.11	21.78	1.04	23.87	1.15
	Vehicle	19.11	0.75	21.76	1.08	23.67	1.04
	Surgery	19.19	0.85	21.87	0.74	23.94	1.34

Table 17: ANOVA statistics for cranial vault height comparing at 10, 25, and 42 days of age.

	10 Days		25 Days		42 Days	
	F Value	P Value	F Value	P Value	F Value	P Value
Phenotype	0.097	0.756	0.405	0.528	0.054	0.817
Treatment	1.020	0.295	0.222	0.802	1.598	0.216
Phenotype*Treatment	0.486	0.618	0.108	0.897	1.301	0.284

3.7 CRANIAL VAULT LENGTH

Figure 16 represents changes in cranial vault means by phenotype and treatment group. Measurements for total cranial vault length were not significant across phenotype or treatment groups for time points 10 day, 25 day and 42 day. See Tables 18 and 19, and Figures 16 and 17.

Table 18: Mean values for cranial vault length at days 10, 25, and 42 of age.

		10 Days		25 Days		42 Days	
		Mean	SD	Mean	SD	Mean	SD
In-Colony Normal	Control	35.91	2.21	41.76	3.58	47.74	1.83
	Vehicle	34.29	2.16	42.09	1.54	47.85	1.73
	Surgery	35.69	1.61	41.88	1.78	46.55	2.60
Delayed Onset	Control	35.13	1.81	41.03	0.99	47.07	1.75
	Vehicle	35.74	2.29	42.25	1.80	48.02	1.43
	Surgery	35.36	1.92	41.53	2.03	46.60	2.12

Table 19: ANOVA statistics for cranial vault length at 10, 25, and 42 days of age

	10 Days		25 Days		42 Days	
	F Value	P Value	F Value	P Value	F Value	P Value
Phenotype	0.032	0.860	0.253	0.618	0.064	0.801
Treatment	0.346	0.710	0.649	0.528	1.859	0.170
Phenotype*Treatment	1.454	0.245	0.213	0.809	0.194	0.824

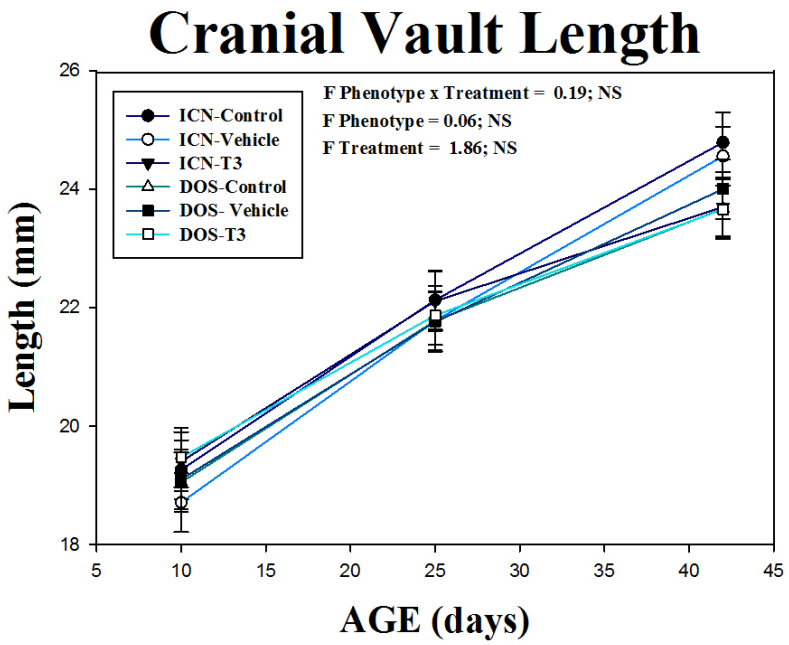

Figure 16: No significant difference in cranial vault lengths amongst the groups.

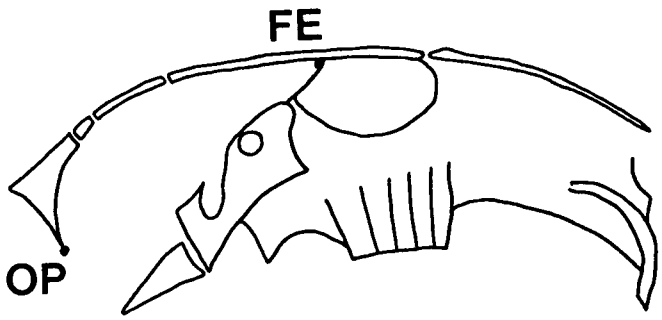

Figure 17: Cranial vault length made by opisthion (OP) and fronto-ethmodale (FE).

3.8 CRANIAL VAULT SHAPE INDEX

Figure 18 represents changes in cranial vault shape index means by phenotype and treatment group. Measurements taken for the cranial vault shape index were all insignificant with the exception at 42 days of age for the treatment groups compared to respective control groups ($F = 5.837$; $p<0.006$). See Tables 20 and 21, and Figures 18 and 19.

Table 20: Mean values for cranial vault shape index at 10, 25, and 42 days of age.

		10 Days		25 Days		42 Days	
		Mean	SD	Mean	SD	Mean	SD
In-Colony Normal	Control	53.75	2.76	53.13	2.51	51.96	1.02
	Vehicle	54.64	2.41	51.80	2.38	49.29	1.95
	Surgery	54.43	3.06	52.78	0.92	50.16	2.24
Delayed Onset	Control	54.25	1.73	53.08	1.80	50.70	1.44
	Vehicle	53.58	2.60	51.53	2.21	49.28	1.86
	Surgery	55.17	3.13	52.71	1.82	51.36	1.32

Table 21: ANOVA statistics for cranial vault shape index at 10, 25 and 42 days of age.

	10 Days		25 Days		42 Days	
	F Value	P Value	F Value	P Value	F Value	P Value
Phenotype	0.006	0.940	0.037	0.849	0.002	0.968
Treatment	0.342	0.712	2.040	0.144	5.837	0.006*
Phenotype*Treatment	0.614	0.546	0.013	0.987	1.433	0.251

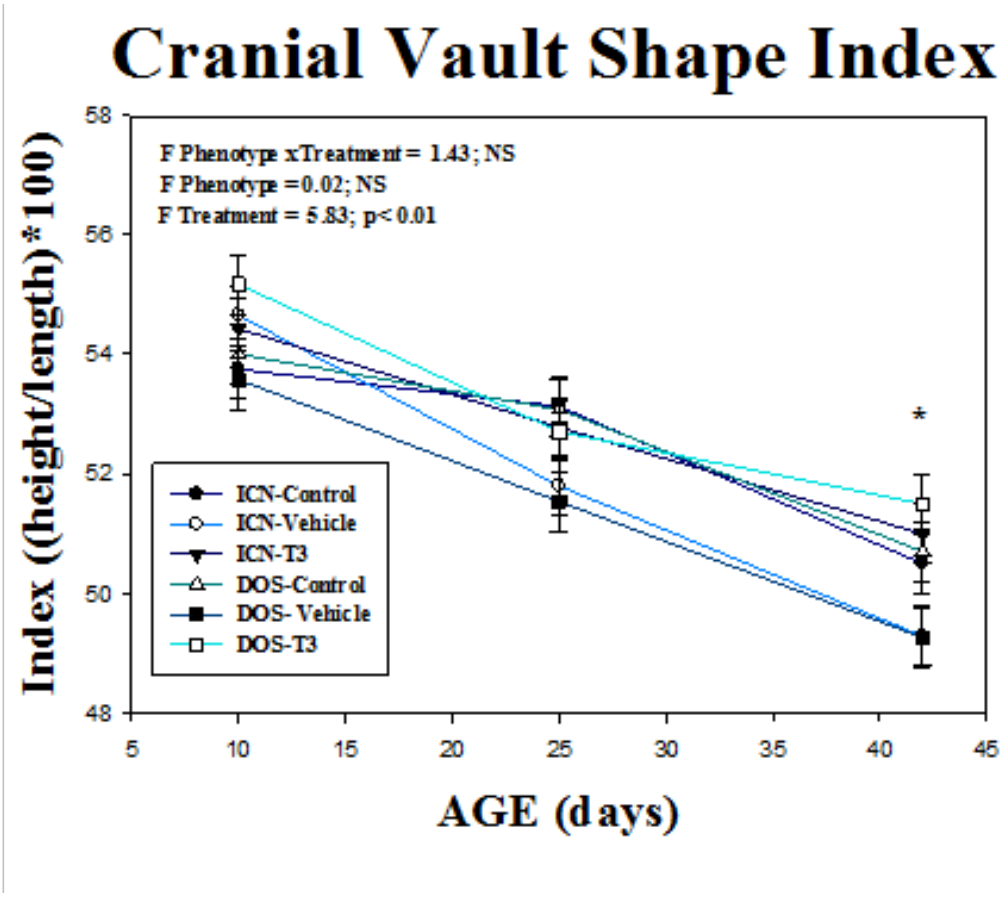

Figure 18: Cranial vault shape index decreased in both treatment groups (T3).

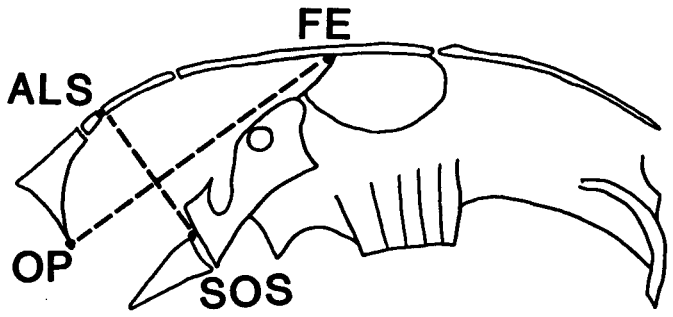

Figure 19: Cranial vault shape index made from intersecting lines of anterior lambdoid suture (ALS) and spheno-occipital suture (SOS) with opisthion (OP) and fronto-ethmodale (FE).

3.9 CRANIAL BASE ANGLE

Figure 20 represents changes in cranial base angle means by phenotype and treatment group. No significant changes in mean cranial base angles were seen between phenotype or treatment groups across time points 10 days, 25 days, or 42 days. See Tables 22 and 23, and figures 20 and 21.

Table 22: Mean values for cranial base angle at 10, 25, and 42 days of age.

		10 Days		25 Days		42 Days	
		Mean	SD	Mean	SD	Mean	SD
In-Colony Normal	Control	178.56	1.16	176.10	1.74	175.56	4.32
	Vehicle	174.81	3.84	177.94	1.60	177.62	1.52
	Surgery	175.44	3.41	179.30	0.43	176.87	2.20
Delayed Onset	Control	175.84	2.91	175.96	3.10	178.36	1.50
	Vehicle	176.53	2.99	176.16	2.41	175.34	4.00
	Surgery	177.07	2.16	176.99	1.21	176.54	3.41

Table 23: ANOVA statistics for cranial base angle at 10, 25, 42 days of age.

	10 Days		25 Days		42 Days	
	F Value	P Value	F Value	P Value	F Value	P Value
Phenotype	0.057	0.813	4.230	0.046	0.005	0.946
Treatment	1.168	0.321	2.700	0.080	0.094	0.910
Phenotype*Treatment	2.792	0.072	0.807	0.453	2.579	0.089

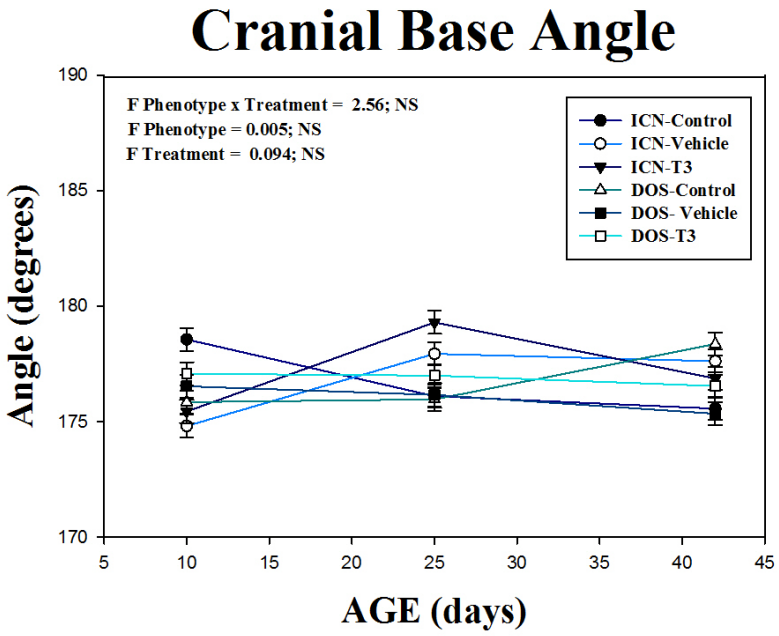

Figure 20: Cranial base angle did not change amongst treatment groups.

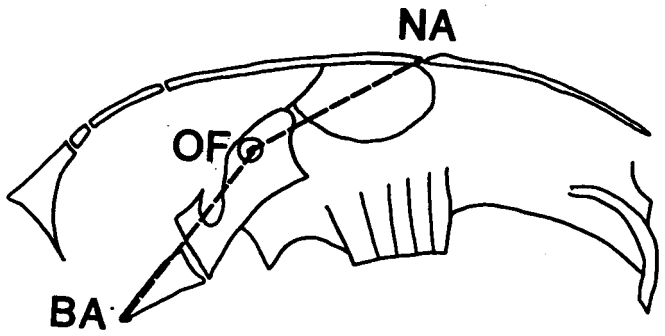

Figure 21: Cranial base angle from basion (BA), optic foramen (OF) and nasion (NA).

3.10 PALATAL ANGLE

Figure 22 represents changes in palatal angle means by phenotype and treatment group. Measurements taken for palatal angle revealed significant changes in 42 day rabbits in the delayed on-set rabbits and also both treatment T3 groups ($F = 4.535$; $p<0.05$; and $F = 3.333$; $p<0.05$). Other measurements taken at time points 10 days and 25 days or between the phenotype-treatment groups did not show any significant differences. See Tables 24 and 25, and Figures 22 and 23.

Table 24: Mean values for palatal angle at 10, 25, and 42 days of age.

		10 Days		25 Days		42 Days	
		Mean	SD	Mean	SD	Mean	SD
In-Colony Normal	Control	128.82	3.08	129.22	2.27	125.10	0.98
	Vehicle	129.15	2.94	128.89	2.76	124.87	2.49
	Surgery	128.29	4.37	128.59	3.09	122.96	4.44
Delayed Onset	Control	129.14	3.86	131.43	2.89	127.77	2.33
	Vehicle	131.77	4.22	130.45	2.94	127.58	4.06
	Surgery	129.63	3.44	129.03	2.82	123.98	2.89

Table 25: ANOVA statistics for palatal angle at 10, 25, and 42 days of age.

	10 Days		25 Days		42 Days	
	F Value	P Value	F Value	P Value	F Value	P Value
Phenotype	1.626	0.209	2.226	0.144	4.535	0.040*
Treatment	0.956	0.392	0.775	0.468	3.333	0.046*
Phenotype*Treatment	0.436	0.650	0.265	0.768	0.291	0.749

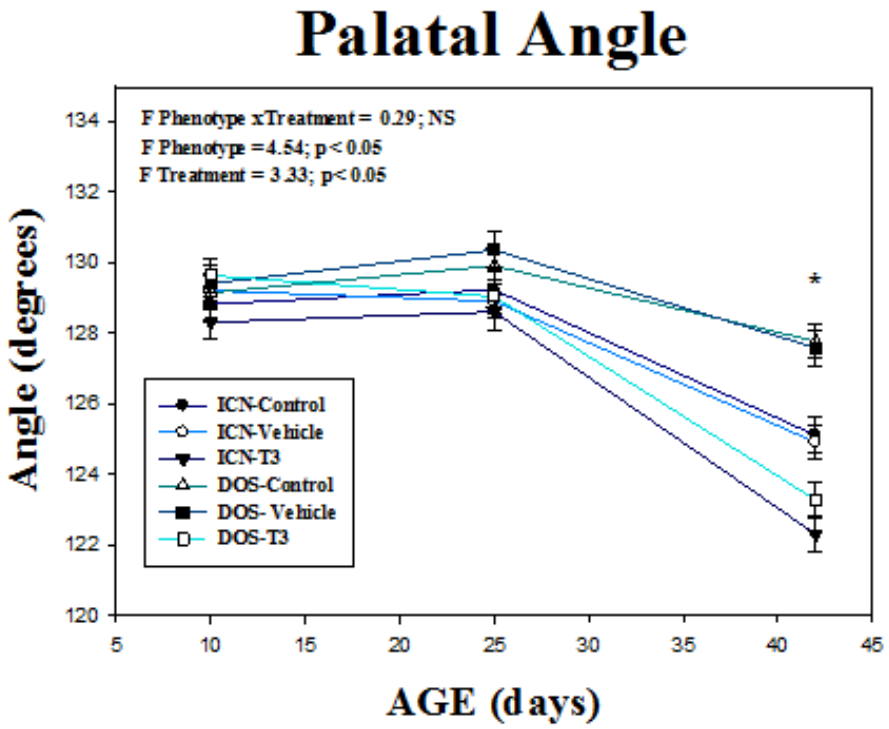

Figure 22: Decreased palatal angles were seen between the two phenotypes and the T3 groups with control groups (T3 rabbits had decreased palatal angles).

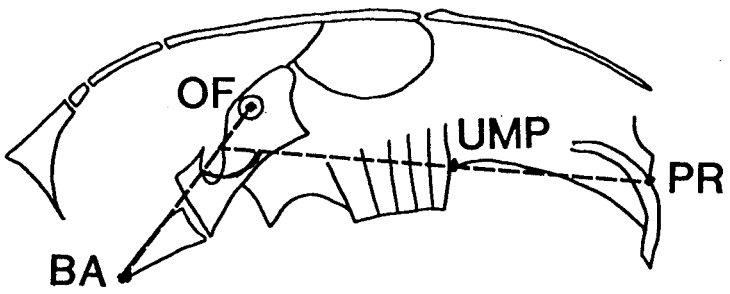

Figure 23: Palatal angle made by intersecting lines of basion (BA) and optic foreman (OF) with upper molar point (UMP) and prosthion (PR).

4.0 DISCUSSION

This study consisted of 65 rabbits from a colony that demonstrates similar phenotypic variability of craniosynostosis to humans. There were 24 in-colony normal rabbits and 41 with delayed on-set craniosynostosis. It was hypothesized that post-natal administration of exogenous tri-iodothyronine (T3) would result in decreased sutural growth than control rabbits in normal or delayed on-set groups. It was further hypothesized that an interaction of the postnatal treatment of exogenous tri-iodothyronine (T3) and a genetic propensity for synostosis would accelerate suture fusion and result in more severe phenotypes in individuals with a synostotic genotype compared to controls.

Results demonstrated that administration of tri-iodothyronine (T3) elevated blood circulating levels of T3 in treatment groups of delayed on-set and in-colony normal rabbits compared to control rabbits. Elevated T3 levels were achieved indicating possible thyrotoxicosis, but in order to diagnosis the rabbits as truly hyperthyroid, it would require clinical assessments and measurements that were not monitored in this study (appetite, physical stamina, etc.) as well as metacarpal growth measurements. Body weights were taken on the rabbits, and treated rabbits with T3 showed significantly decreased body weight compared to control rabbits, which may indicate that the rabbits might be hyperthyroid. In order to know for certain that the body weights decreased due to administered T3, it would be necessary to identify the somatic growth for each rabbit. This would rule out any low weights due to normal variation in somatic growth rate of the

rabbits (assuming chronologic dates of the rabbits do not indicate actual growth status). Decreased T4 levels in treated rabbits show that the rabbits' endocrine response received a negative feedback from administered T3 levels, shutting off endogenous T4 production which also supports that the T3 treated rabbits exhibited thyrotoxicosis.

The effects of T3 on treated rabbits resulted in decreased body weights, decreased cranial vault shape index, and decreased palatal angle. What wasn't significant, were any changes in total coronal marker separation, total cranial length, total cranial height, total craniofacial length, and cranial base angle. Even though changes in coronal marker separation were not significant by treatment, the differences between the phenotypes were significant, indicating that delayed on-set rabbits demonstrate less coronal suture growth than their in-colony normal littermates. Although, administration of T3 to either DOS or ICN did not produce significantly different marker separation from control groups, there was a trend towards decreased marker separation that is visually noted in Figure 4. Due to the small sample size of each treatment group, the relative late administration of T3, and the small treatment effect, the significance of the treatment outcome could be compromised. Perhaps, increasing the sample size, and thereby increasing the power of the study, would result in statistical significance. Decrease in the coronal marker separation would foretell reduced anterior-posterior growth of the cranium, thereby requiring compensatory growth in other dimensions. Cranial esthetic disturbances would be likely, in addition to an impact on mental acuity, eyes, etc.

A decrease in coronal marker separation is not the only variable that could indicate a change in the cranial growth pattern, since a decrease of growth in one suture could likely involve compensatory growth in another suture. Coronal marker separation was only slightly (not significantly) reduced from T3 administration, and there are other variables that did show

significant growth changes in treatment groups when compared to control groups. A decrease in the cranial vault shape index means that the ratio of total cranial length/total cranial height has decreased. That means that total cranial height (TCH) increased, while the total cranial length (TCL) decreased. Both these variables (TCH, TCL) were not significantly changed of themselves, but their subtle differences found in their ratio were significant. This change of growth pattern, towards an increased superior-inferior pattern, is consistent with decreased coronal suture growth and decreased anterior-posterior growth (see Figure 13).

Although there was a decrease in the cranial vault shape index, suggesting an increased cranial height, there was not an increase in the craniofacial length. This may be due to the fact that the craniofacial length in rabbits is comprised of their cranium and snout, including the frontonasal growth apparatus. The phenotypic presentation of craniosynostosis in the colony of rabbits used in this study involves primarily the coronal suture, and no other cranial sutures. That means the frontonasal suture is not affected, and growth of the frontonasal suture should be uninhibited. Thus, craniofacial length would not be as grossly affected as the isolated measure of total cranial length (TCL). Given that the TCL was not statistically reduced in this study, it would lead one to expect that the craniofacial length would also not be reduced (as was observed in this study).

A decrease in cranial base angle was not observed in this study, but a decrease of the palatal plane angle was observed. A severely affected rabbit with coronal synostosis would show exacerbated cranial growth compensations, including a rotation of the cranial base angle and likely mid-face disturbances seen in the maxilla. Treated rabbits with T3 in this study did show a change of the palatal plane angle. And although a similar significant change in the cranial base angle was not observed, that change may exist in a study with an increased sample size.

Change in the cranial growth pattern from administration of T3 has shown an impact in the 2-dimensional variables measured on the lateral cephalograms. In order to further investigate the impact of thyroid hormones on the cranial development, measuring the change in bizygomatic width from dorsoventral radiographs would elucidate a change in a different 2-dimensional plane, of the cranial growth disturbances from administered T3. Ideally, growth studies of administered exogenously T3 would further benefit from 3-dimensional diagnostic imaging that could better relate to the clinical presentation of craniosynostosis.

The effects of exogenously administered thyroid hormone, in the case of this study tri-iodothyronine (T3), had no statistical difference between the two phenotypes. We observed decreased body weights for T3 treated delayed on-set rabbits in-colony normal rabbit groups. However, the difference between the two treatment effects in these two groups was not statistically significant. This indicates that there was no gene-environment interaction of T3 on the gene(s) that are causing coronal synostosis in this colony of rabbits. A gene interaction could still exist due to two confounding variables in this study: 1) the in-colony normal rabbits are genetically similar to the delayed on-set rabbits, and that a gene-environment interaction is occurring in both phenotypes. The gene in question could be either the same or different gene (s) than the gene(s) causing synostosis. Including a wild type liter into the study would control for the genetic presentation of the in-colony rabbits: 2) the statistical differences between the two treatment groups are currently not significant, but if the sample size increased to account for a power suitable for the treatment effect, then a gene-environment interaction between the two phenotypes be observed.

5.0 CONCLUSION

It appears the dosage used of T3, 0.2mg/kg, created a thyrotoxicosis in the rabbits, as seen by the increased T3 blood serum levels, the decreased levels of circulating endogenous T4, and the decreased body weights following treatment. The effect of T3 on cranial development showed changes in cranial vault shape index and palatal plane angle. Although the decreased coronal suture marker separation observed in T3 treated rabbits was not statistically significant, that separation may become significant with an increased sample size. No gene/environment interaction was observed in this study and other factors affecting variable phenotypic expression should be explored.

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